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Microbial adhesion to surface-grafted polyacrylamide brushes after long-term exposure to PBS and reconstituted freeze-dried saliva

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Abstract: Polyacrylamide (PAAm) brushes, covalently grafted from silicon wafer surfaces were examined for their ability to inhibit microbial adhesion after long-term exposure to PBS or reconstituted freeze-dried saliva for time intervals from 48 h up to 1 month at 37°C. Microbial adhesion after exposure was studied in a parallel plate flow chamber. Infrared spectra showed that PAAm brushes exhibit good chemical stability upon incubation in both PBS and reconstituted freeze-dried saliva up to 1 month. Reductions in microbial adhesion on

PAAm brushes after exposure to PBS or reconstituted freeze-dried saliva varied from 63 to 93% depending on the microbial strain considered, even after 1 month of exposure of the brushes to reconstituted freeze-dried saliva. © 2010 Wiley Periodicals, Inc. *J Biomed Mater Res Part A* 94A: 997–1000, 2010.

Key Words: polyacrylamide brush, biomedical applications, exposure to physiological fluids, long time stability, microbial adhesion

INTRODUCTION

Microbial adhesion to biomaterial surfaces is the first step in the development of a biomaterial-associated infection.¹ Polymer brushes not only withstand protein adsorption but also microbial adhesion, although the ability of polymer brushes to reduce microbial adhesion does not necessarily correlate with the ability to reduce protein adsorption.² Polymer brushes reduce adhesion of microorganisms as they constitute an entropic barrier and decrease attractive Lifshitz-Van der Waals forces between the microorganisms and an implant surface.^{3–9} Despite the fact that polymer brushes are designed to withstand adhesion of microorganisms and adsorption of proteins, adsorption of small proteins to polymer brushes may occur and interfere with a brush's resistance against microbial adhesion. Protein adsorption to surfaces carrying poly(ethylene glycol) (PEG) brushes has been described to occur through three distinctly different modes^{10,11}: (i) Primary adsorption involving direct, attractive contact with the substratum surface, (ii) Secondary adsorption at the outer edge of the brush due to weak Lifshitz-Van der Waals attraction, and (iii) Ternary adsorption of proteins within the brush itself as a result of weak PEG-protein attraction. Clearly, the mode of adsorption will depend on the polymer grafting density and the length of the polymer chain, as well as on the size of the adsorbing proteins in their native state.^{12–14} Protein adsorption at the brush edges has been demonstrated to decrease with the length of oligo(ethylene oxide) chains covalently bound to the surface.¹⁵

The ability of polymer brush coatings on biomaterial surfaces to reduce microbial adhesion should not be impaired by long-term exposure to biological fluids. Polyethylene oxide (PEO) brushes adsorbed to glass surfaces by reaction of terminal vinyl groups were stable and effective against microbial adhesion after exposures up to only 4 h in saliva and up to 36 h in urine.¹⁶ The complete loss of polymer chains with a thickness of 3.7 nm from the surface took place on average after 48 h, as determined by X-ray photoelectron spectroscopy. Short (2.5 nm) PEG brush coatings covalently bound to silicon wafers and glass surfaces¹⁷ were stable in phosphate-buffered saline (PBS, pH 7.4) at 37°C up to 24 days of incubation *in vitro*. However, degradation of the PEG film thickness occurred rapidly after day 25 with a complete loss of the film after 27 days. Comb-copolymers such as poly(L-lysine)-*g*-poly(ethylene glycol)¹⁸ grafted covalently on a silicon wafer surface through an aldehyde plasma treatment, gave higher stabilities under extreme pH values or high ionic strength. Covalent binding of long polymer chains lead to a very good resistance against microbial adhesion and growth. Zwitterionic poly(carboxybetaine methacrylate) (pCBMA) covalently grafted (length 29 nm) from glass surfaces via atom transfer radical polymerization (ATRP) achieved 95% reduction in colonization by *Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* 239 colonization up to 10 days.¹⁹

Long (20 nm) polyacrylamide (PAAm) brushes, attached to a silicon wafer surface via chemical bonds using an ATRP technique, with a high grafting density of about 0.6 nm^{–2}

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were also demonstrated to possess good resistance against microbial adhesion.²⁰ By using the same ATRP method, PAAm brushes were grafted from the surface of silicone rubber and these brushes showed minimal protein adsorption from a protein-rich fluid-like saliva.²¹ Biological fluids including saliva contain a mixture of different small and large proteins^{22–25} and it is unclear how long-term-exposure of PAAm brushes to a mixture of different proteins affects the efficacy of PAAm brushes to resist microbial adhesion. Therefore, in this study, PAAm brushes, covalently grafted from silicon wafer surfaces were examined for their ability to inhibit microbial adhesion after long-term exposure to PBS and reconstituted freeze-dried human saliva for time intervals from 48 h up to 1 month at 37°C.

MATERIALS AND METHODS

Materials

Silicon wafers (125 mm diameter, 900 μm thick, both sides polished, 1-1-1 orientation, and phosphorus doped to 1000 $\Omega\text{ cm}$ resistivity) were supplied by Topsil Semiconductors Materials A/S (Frederikssund, Denmark) and cut into 2.5 cm \times 2 cm samples. Calcium chloride, sodium chloride, potassium chloride, potassium phosphate, sodium azide, and phenylmethylsulfonylfluoride were received from Merck. All solvents were reagent grade and used without further purification.

Brush preparation

The organic contaminations of the silicon wafer surface were removed by exposing the surface in an UV/ozone reactor for 30 min.²⁰ Subsequently, surface modification was done following the reaction procedure as described previously.¹⁹ First, aminosilanization was carried out in a toluene solution (2% v/v) of γ -aminopropyltriethoxysilane. Second, the amino groups were reacted with 4-(chloromethyl)benzoyl chloride in dichloromethane solution (2% wt/v) to introduce the initiator and, finally, the atom transfer radical polymerization (ATRP) of acrylamide was carried out as described before.²⁰ The brush length and grafting density was calculated using ellipsometry as described previously.²⁰

Collection of saliva and exposure of PAAm brushes

From at least 20 healthy volunteers of both sexes, human whole saliva was collected into ice-chilled cups. After the saliva was pooled and centrifuged at 10,000g for 5 min at 10°C, phenylmethylsulfonylfluoride was added to a final concentration of 1 mM as a protease inhibitor. The solution was again centrifuged, dialyzed overnight at 4°C against demineralized water, and freeze-dried for storage. A solution of 1.5 mg/mL of freeze-dried saliva in adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride, and 1 mM calcium chloride, pH 6.8) will be denoted in this article as reconstituted freeze-dried saliva. Reconstituted freeze-dried saliva was supplemented with 0.02% sodium azide to prevent bacterial growth. All volunteers gave their informed consent to saliva donation, in accordance with the rules set out by the Ethics Committee at the University Medical Center Groningen.

Silicon wafers with and without PAAm brushes were placed in PBS or reconstituted freeze-dried saliva (without stirring) for 48 h or 1 month, at 37°C. Subsequently, samples were rinsed with a flow of demineralized water for 5 min and used for microbial adhesion or surface chemical characterization.

Physico-chemical brush characterization before and after exposure to PBS or reconstituted freeze-dried saliva

Silicon wafers with and without a PAAm brush before and after exposure to PBS and reconstituted freeze-dried saliva were analyzed with Fourier transform infrared spectroscopy. Transmission FTIR measurements were performed on a Bruker IFS 66v/S spectrometer equipped with a DTGS detector. All spectra are averages of 100 scans measured at a resolution of 4 cm^{-1} . The spectrum of a clean silicon wafer was used as a reference. Before measurements, silicon wafer surfaces with and without a PAAm brush exposed to PBS and reconstituted freeze-dried saliva were rinsed with demineralized water for 5 min and dried under vacuum. Opus[®] software was used to integrate the peak areas by using the integration method D between the limits 1627 cm^{-1} and 1530 cm^{-1} with a horizontal baseline set at 1753 cm^{-1} .

Microbial adhesion

Two bacterial strains (*Staphylococcus aureus* ATCC 12600 and *Streptococcus salivarius* GB 24/9) and one yeast strain (*Candida albicans* GB 1/2) were used to evaluate the inhibitory effects of the brushes on microbial adhesion under moderate flow (10 s^{-1}). All strains were cultured as detailed before.²⁰ Microbial adhesion to noncoated and PAAm brush-coated silicone wafer was observed in a parallel plate flow chamber as reported previously.²⁰

RESULTS AND DISCUSSION

Physico-chemical characterization of a silicon wafer with and without a PAAm brush before and after exposure to PBS and reconstituted freeze-dried saliva

FTIR spectra of PAAm brush-coated silicon wafers before and after exposure to PBS for 1 month at 37°C are presented in Figure 1. PAAm shows characteristic absorption peaks at 3334 cm^{-1} and 3198 cm^{-1} , attributed to the N—H stretching modes of the amide functionality and bands at 1660 cm^{-1} and 1617 cm^{-1} assigned to the amide C=O stretching mode, respectively [Fig. 1(a)]. The differences in the carbonyl (1660 cm^{-1}) peak areas in the PAAm transmission spectra before and after the exposure to PBS [Fig. 1(b)] are only 4% as determined with the Opus[®] software and not significantly different. This indicates that PAAm brush degradation did not occur after the exposure to PBS for 1 month. Thus according to our knowledge, the chemical stability of our polymer brush coatings in PBS is the longest reported in literature hitherto.

Also the carbonyl (1660 cm^{-1}) peak area from PAAm spectrum after exposure to reconstituted freeze-dried saliva for 48 h [Fig. 2(b)] differs only 7% from the area of the same peak from PAAm spectrum before exposure to

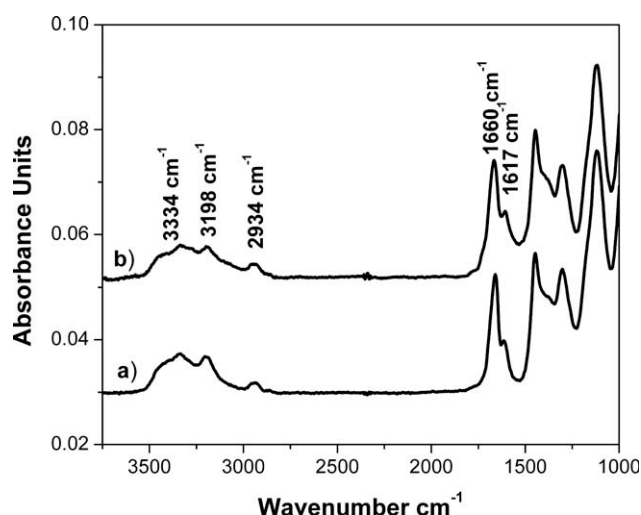


FIGURE 1. FTIR spectra of PAAm-coated silicon wafer (a) before exposure to PBS and (b) after exposure to PBS for 1 month. The spectra are Y-shifted for clarity.

reconstituted freeze-dried saliva, which is not a significant difference. The characteristic peaks for PAAm in Figure 2(c) are somewhat deformed when compared with those in Figure 2(a), which is an indication of a deposition of a very thin layer of salivary proteins.

Microbial adhesion to the PAAm brush after exposure to PBS and reconstituted freeze-dried saliva for 48 h and 1 month

Figure 3 presents the adhesion of two bacterial and one yeast strain to uncoated and brush-coated silicon wafers after exposure to PBS and reconstituted freeze-dried saliva for 48 h and 1 month at 37°C. All brush-coated samples exposed to both PBS and reconstituted freeze-dried saliva reduce microbial adhesion.

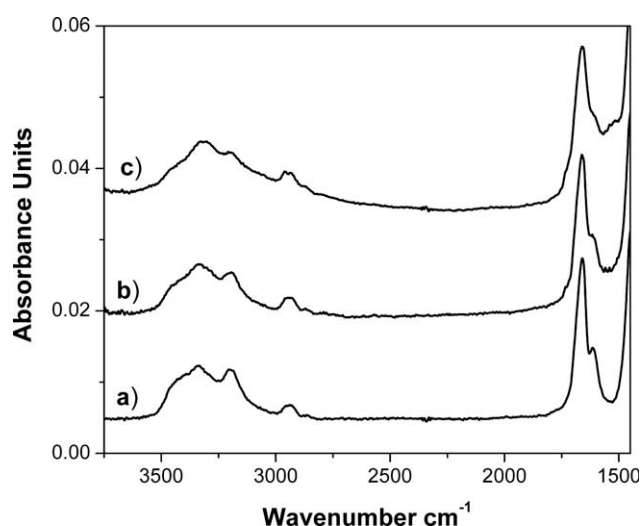


FIGURE 2. FTIR spectra of a PAAm-coated silicon wafer (a) before exposure to saliva, (b) after exposure to saliva for 48 h, and (c) after exposure to saliva for 1 month. The spectra are Y-shifted for clarity.

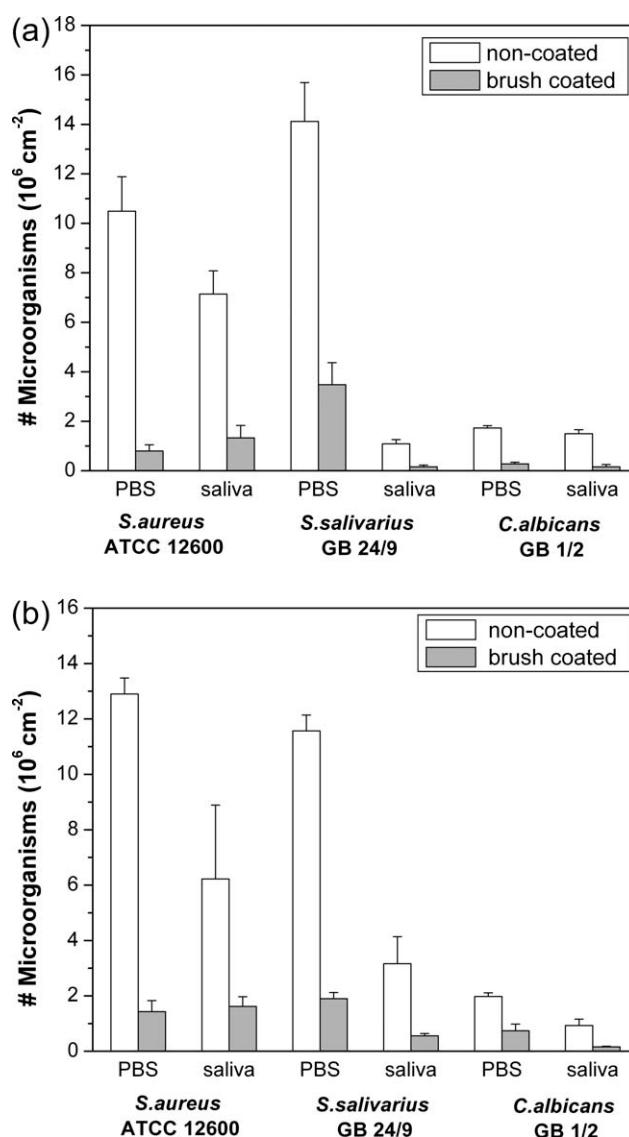


FIGURE 3. Microbial adhesion to uncoated and PAAm brush-coated silicon wafers after exposure to PBS and saliva for 48 h (a) and 1 month (b) 37°C. All error bars represent the average standard deviation obtained from three separate experiments.

S. aureus adhesion on brushes exposed to PBS is similar to its adhesion on brushes exposed to reconstituted freeze-dried saliva. However, on noncoated silicon wafers exposed to PBS, *S. aureus* adheres in higher number than on samples exposed to reconstituted freeze-dried saliva. The reduction of *S. aureus* adhesion after 48 h to the brush is higher for samples exposed to PBS ($91\% \pm 3\%$) than to reconstituted freeze-dried saliva ($78\% \pm 5\%$). Very low adhesion of *S. salivarius* takes place on both brush-coated and uncoated wafers after salivary protein adsorption. However, its adhesion to brushes exposed to reconstituted freeze-dried saliva after 48 h ($0.2 \times 10^6 \text{ cm}^{-2}$) is much lower than to the brush exposed to PBS ($3.5 \times 10^6 \text{ cm}^{-2}$). PAAm brushes exposed to reconstituted freeze-dried saliva and PBS equally reduce adhesion of *C. albicans*.

It is very important to notice that PAAm brushes remain effective even after 1 month of incubation in PBS or reconstituted freeze-dried saliva [Fig. 3(b)]. Reductions in microbial adhesion after 1 month remained high between 63% and 89%. The adhesion of *S. salivarius* and *C. albicans* on samples exposed to reconstituted freeze-dried saliva was even further reduced compared with their adhesion on samples exposed to PBS.

Taking into account that the time interval to study the protein adsorption is much longer compared with other studies in literature, the high stability of PAAm polymer brush coatings and their reduction of microbial adhesion after exposure to reconstituted freeze-dried saliva is not only of scientific interest but also holds promise for application of these coatings in the human body.

CONCLUSIONS

Long (20 nm) PAAm brushes grafted covalently from silicon wafer surfaces were chemically stable and remained effective against microbial adhesion even after 1 month of exposure to PBS or massive protein challenge from reconstituted freeze-dried saliva at 37°C. In line, the PAAm brush attracted little adhesion of *S. aureus* ATCC 12600, *S. salivarius* GB 24/9, and *C. albicans* GB 1/2, also after exposure to PBS or reconstituted freeze-dried saliva for 1 month. The grafting density, the polymer thickness, and the attachment mode of the polymer chains on the surface are suggested to be the key in the success of the coating performance and stability. PAAm brushes are thus promising coatings for biomedical purposes with long-term antiadhesive properties against a variety of microbial strains and species.

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